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genesis 44:465–476 (2006)

ARTICLE

Site-Specific Recombination Systems for the Genetic Manipulation of Eukaryotic Genomes

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Received 22 May 2006; Revised 24 July 2006; Accepted 8 August 2006

Summary: Site-specific recombination systems, such as the bacteriophage Cre-*lox* and yeast FLP-*FRT* systems, have become valuable tools for the rearrangement of DNA in higher eukaryotes. As a first step to expanding the repertoire of recombination tools, we screened recombination systems derived from the resolvase/invertase family for site-specific recombinase activity in the fission yeast *Schizosaccharomyces pombe.* Here, we report that seven recombination systems, four from the small serine resolvase subfamily (CinH, ParA, Tn1721, and Tn5053) and three from the large serine resolvase subfamily (Bxb1, TP901-1, and U153), can catalyze site-specific deletion in *S. pombe*. Those from the large serine resolvase subfamily were also capable of site-specific integration and inversion. In all cases, the recombination events were precise. Functional operation of these recombination systems in the fission yeast holds promise that they may be further developed as recombination tools for the site-specific rearrangement of plant and animal genomes. genesis 44:465–476, 2006. **Published 2006 Wiley-Liss, Inc.**†

Key words: excision; inversion; integration; recombinase; resolvase; integrase

INTRODUCTION

Two decades have passed since Sauer (1987) showed that the Cre-*lox* site-specific recombination system functions in yeast. Since then, the Cre-*lox* system has been developed into one of the most important tools for site-specific genome engineering (Branda and Dymecki, 2004; Nagy, 2000; Ow, 2002; Sorrell and Kolb, 2005; van der Weyden *et al*., 2002). Other recombination systems have also been investigated for the rearrangement of DNA in heterologous hosts. To date, there are over a dozen recombination systems described to function in higher eukaryotes, which can be divided into several groups. The first group is comprised of Cre-*lox* (Sauer and Henderson, 1988), FLP-*FRT* (Golic and Lindquist, 1989), R-*RS* (Onouchi *et al*., 1991), and Gin-*gix* (Maeser and Kahmann, 1991), where Cre, FLP, R, and Gin are the recombinases and *lox*, *FRT*, *RS*, and *gix* are the respective recombination sites. In these systems, the genetic cross over between recombination sites regenerates a site of identical or highly similar in sequence, and the recombination reaction is fully reversible. In a second group, represented by β-*six* (Diaz *et al*., 2001) and γδ-*res* (Schwikardi and Droge, 2000), where β and γδ are resolvases, and *six* and *res* the respective recombination sites, the reaction also regenerates a product site of the same sequence. However, since the reaction catalyzes only intra-molecular excision but not inter-molecular cointegration, an excision event cannot be reversed. In a third group, represented by phiC31 (ϕC31) (Groth *et al*., 2000; Thomason *et al*., 2001), R4 (Olivares *et al*., 2001), TP901-1 (Stoll *et al*., 2002), λ (Christ and Droge, 2002), HK022 (Kolot *et al*., 2003), ϕBT1 (Chen and Woo, 2005), Bxb1 (Keravala *et al*., 2006; Russell *et al*., 2006), A118, U153, ϕFC1, and ϕRV1 (Keravala *et al*., 2006), the recombinase acts on two recombination sites that differ in sequence, typically known as attachment sites *attB* and *attP*, to yield product sites known as *attL* and *attR*. The reaction can be excision, inversion, or integration, and is not reversible unless an additional protein, an excisionase, is provided.

Our laboratory is interested in developing additional recombination systems for use in plants to supplement the few efficient systems currently available, as well as to provide public access to tools for the genetic improvement of crop varieties. The excision of transgenic DNA has many applications for the commercial development of transgenic plants. For example, the removal of an antibiotic resistance gene eliminates the possibility of its horizontal transfer, as well permits that same selection gene to be used again for subsequent rounds of gene transfer (Dale and Ow, 1991; Russell *et al*., 1992). Site-specific deletion of marker genes from major crop plants such as corn, rice, wheat, cotton, and soybean has been

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Published online in
Wiley InterScience (www.interscience.wiley.com).
DOI: 10.1002/dvg.20237

achieved (Gilbertson *et al*., 2003), and a high lysine corn line from Monsanto and Cargill, which used Cre-*lox* to excise away the kanamycin resistance marker, has received U.S. regulatory approval for commercial cultivation. Site-specific DNA integration also holds promise for expediting the genetic improvement of crop varieties. Gene transfer via site-specific integration permits a higher rate of transformants with a precise single-copy of the introduced DNA (Albert *et al*., 1995). More importantly, this process leads to a higher rate of predictable gene expression (Day *et al*., 2000; Srivastava and Ow, 2002; Srivastava *et al*., 2004). Other applications of site-specific recombination include the precise reconstruction of viral expression vectors (Marillonnet *et al*., 2004) and the targeted mutagenesis of plant chromosomes (Zhang *et al*., 2003).

To provide additional DNA manipulation tools for plant genetic modification, a collection of prokaryotic recombination systems were tested for function in the fission yeast *Schizosaccharomyces pombe*. In the screen for dedicated deletion systems, functional operation was found for 4 DNA excision systems, CinH, ParA, Tn1721, and Tn5053. These systems belong to the small serine resolvase subfamily. Each of these systems catalyzes recombination between identical recombination sites. However, since they do not catalyze intermolecular reactions, the excision reaction cannot be reversed. The CinH system is derived from *Acetinetobacter* plasmids pKLH2, pKLH204, and pKLH205 (Kholodii, 2001). The 189 aa CinH recombinase recognizes a 113 bp recombination site known as *RS2*. The ParA system is from a plasmid operon *parCBA*, which is responsible for the maintenance of broad host range plasmids RK2 and RP4. The 222 aa ParA recombinase recognizes a 133 bp recombination site termed *MRS* (multimer resolution site) (Gerlitz *et al*., 1990). The Tn1721 system is derived from the 3.8 kb transposon Tn1721 (Rogowsky and Schmitt, 1985). The *tnpR*-encoded 186 aa recombinase recognizes a 120 bp recombination (*res*) site. The Tn5053 system is derived from a 8.4 kb transposon by the same name (Kholodii *et al*., 1995). Recombination between two 176 bp recombination (*res*) sites is mediated by the 204 aa *tniR*-encoded recombinase.

In the screen for dedicated integration systems, functional operation was detected with 3 members of the large-serine resolvase subfamily, Bxb1, TP901-1, and U153. In each of these systems, site-specific integration can be catalyzed by a recombinase but excision requires an additional excisionase protein. The Bxb1 system is from the *Mycobacterium smegmati* bacteriophage Bxb1 (Mediavilla *et al*., 2000). The 500 aa Bxb1 recombinase acts on minimal recombination (attachment) sites *attP* and *attB* that are 39 bp and 34 bp, respectively. Recent studies on the Bxb1 system have shown that it could catalyze site-specific recombination in vitro in the absence of secondary proteins or high-energy cofactors (Kim *et al*., 2003). The TP901-1 system is from the temperate bacteriophage TP901-1 of *Lactococcus lactis* subsp. *cremoris* 901-1. The 485 aa TP901-1 recombinase is sufficient to integrate plasmids containing the *attP* site into an *attB* site in the *Lactococcus lactis subsp. Cremoris genome* (Christiansen *et al*., 1996). The minimal sizes of the *attP* and *attB* sites appear to be 56 bp and 43 bp, respectively (Breuner *et al.*, 2001). The U153 system is from *Listeria monocytogenes* bacteriophage U153 (phiCU-SI153/95). The 452 aa U153 recombinase, related to those of ϕC31, TP901-1 and R4, recombines a 57 bp recombination (attachment) site, *attP*, with a 51 bp recombination (attachment) site, *attB*. Integration of plasmid DNA containing *attP* into the bacterial *attB* target has been shown (Lauer *et al.*, 2002). The TP901-1, Bxb1, and U153 systems have since been reported to function in mammalian cells (Keravala *et al*., 2006; Russell *et al*., 2006; Stoll *et al*., 2002).

RESULTS AND DISCUSSION

Site-Specific Excision of Plasmid DNA in *S. pombe*

To determine whether a prokaryotic recombination system has activity in a eukaryotic cell, a plasmid-based assay was used to screen for recombinase-mediated excision events in *S. pombe.* This assay uses a detection plasmid to identify site-specific recombination caused by a co-transformed recombinase-expression plasmid. The His^+ detection plasmid, pPB-X, where "X" denotes the particular recombination system (Fig. 1a), contains a set of system-specific recombination sites flanking an *eGFP* (enhanced green fluorescence protein) coding region situated between a thiamine-repressible promoter, P^{NMT}, and an *ura4* coding region. A corresponding Leu^+ recombinase-expression plasmid, pNMT-X, where "X" denotes the particular recombinase, places the recombinase coding-region under the control of a thiamine-repressible P^{NMT} promoter (Fig. 1b). The plasmids were co-introduced into the $Ade^-Leu^-His^-Ura^-$ *S. pombe* strain FY527 and selected for on leucine- and histidine-deficient media. In the case of successful site-specific excision, recombinase produced from pNMT-X recombines the set of recombination sites in pPB-X. Excision of the *eGFP* fragment would fuse P^{NMT} with *ura4* to form the derivative construct pPBexc-X (X denotes the particular recombination system, Fig. 1c). Hence, the experimental design was intended to provide a phenotypic assay for site-specific excision, the loss of *eGFP* expression concomitant with the gain of Ura^+ prototrophy. This expectation, however, assumes that the recombination site downstream of P^{NMT} does not interfere with expression of the downstream coding region, *eGFP* or *ura4*. Whether that holds true depends on the sequence of the given recombination site.

In addition to the phenotype assay, site-specific recombination was also assayed biochemically by PCR analysis on individual Leu^+His^+ colonies. A primer pair, ***a*** and ***b***, corresponding to the P^{NMT} promoter and the *ura4* ORF is expected to amplify a product of ~1.4 kb in pPB-X, but a smaller product of ~0.74 kb from the exci-

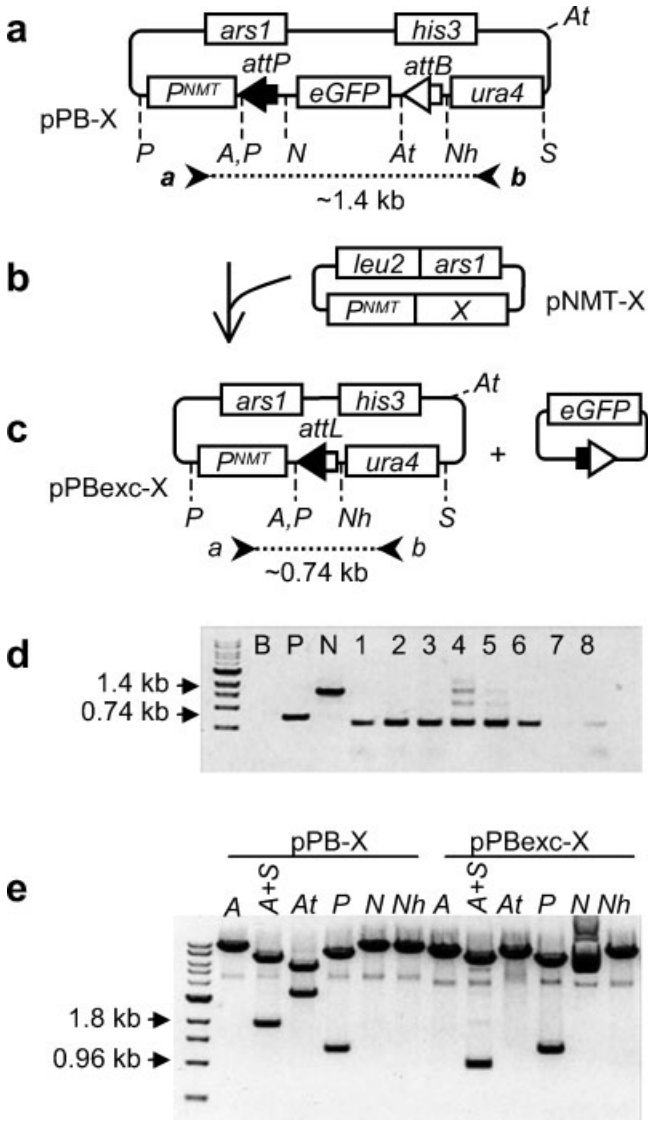

FIG. 1. Site-specific excision of plasmid DNA. (**a**) pPB-X with *eGFP* ORF flanked by sites of recombination system "X", oriented for deletion of *eGFP*. (**b**) Recombinase provided by co-introduced pNMT-X. (**c**) Excision products pPBexc-X and circular *eGFP* fragment. Primers ***a*** and ***b*** (small arrowheads) to amplify PCR product of ~1.4 kb or ~0.74 kb, respectively, before or after *eGFP* excision. (**d**) PCR products from CinH system. Lanes: B, blank; P, positive control from Cre-*lox* system; N, negative control without pNMT-X; lanes 1–8, representative Leu^+His^+ colonies. (**e**) pPBexc-CinH from *S. pombe* retransformed into *E. coli* for *in vitro* cleavage by *Asc*I (*A*), *Asc*I and *Sac*I (*A*+*S*), *Pst*I (*P*), *Aat*II (*At*), *Not*I (*N*), *Nhe*I (*Nh*).

sion derivative pPBexc-X (Fig. 1a,c). The exact size of the PCR product would depend on sizes of the recombination sites of the various systems tested, ranging from 34 to 176 bp. For each recombination system, three independent transformation experiments were conducted, in which 10 Leu^+His^+ colonies from each experiment were analyzed by PCR. Among the colonies that scored positive for excision, representative excision products (pPBexc-X) were retrieved from *S. pombe* and retransformed into *E. coli.* DNA from these pPBexc-X constructs was cleaved with the set of restriction enzymes shown in Figure 1e. Finally, the fragments containing the recombination junctions were sequenced.

Table 1 summarizes the data of the 7 recombination systems compared with the control systems Cre-*lox* and ϕC31. The rate of transformation (mean: 0.05% ± 0.03%) and the recovery of Leu^+His^+ colonies (mean: 1300 ± 340) were similar for all sets of pPB-X + pNMT-X plasmids. With the phenotypic assay, the Cre-*lox* plasmids worked as expected. Transformation of *S. pombe* by pPB-Cre yielded colonies that expressed *eGFP*, but were not Ura^+. Cotransformation with pPB-Cre and pNMT-Cre yielded Leu^+His^+ colonies that were also Ura^+. Likewise, the same held true with the systems TP901-1 and U153. However, with the other systems, the pPB-X constructs did not express *eGFP*, and the Leu^+His^+ colonies were Ura^-. This suggested a possible interference with expression of the reporter gene by the upstream recombination sites.

Despite the lack of a phenotypic assay for many of these systems, the PCR assay was informative. For each of the systems listed in Table 1, nearly all of the 30 Leu^+His^+ colonies examined yielded a PCR product indicative of a deletion event, with a distinct shift in band size from ~1.4 to ~0.74 kb. Although the PCR assay was not meant to be quantitative, the relative intensities between the ~1.4 kb and ~0.74 kb bands provides a rough estimate of the relative efficiency of excision. Compared with the Cre-*lox* reaction, the Tn1721, Tn5053 and U153 systems appear to be significantly less efficient.

The pPBexc-X constructs retrieved from these colonies showed the restriction pattern predicted for an excision event when cleaved with *Asc*I, *Asc*I+*Sac*I, *Aat*I, *Pst*I, *Not*I, or *Nhe*I. In particular, the cleavage product by *Asc*I+*Sac*I shifted from ~1.8 kb in pPB-X to ~0.96 kb in pPBexc-X. When representative recombination junctions were sequenced, in all cases, the recombination events were precise. We conclude that each of these recombination systems is capable of conservative site-specific deletion in *S. pombe*, although in rare instances, Leu^+His^+ colonies were recovered that did not experienced site-specific recombination. These clones were not further examined, as they were presumed to represent aberrant events, such as mutations that may have occurred in the recombinase gene or in the recombination sites of the introduced plasmids.

Site-Specific Inversion of Plasmid DNA in *S. pombe*

To test for DNA inversion, the set of pPB-X constructs (Fig. 1a) were modified such that the *ura4* proximal recombination site was placed in the opposite orientation (Fig. 2a). Site-specific recombination with this set of pPBi-X constructs would be expected to invert the intervening *eGFP* fragment. As before, each pPBi-X plasmid was co-transformed into *S. pombe* FY527 along with pNMT-X. PCR was used to screen the Leu^+His^+ transformants for inversion of the *eGFP* intervening DNA. The primers used

Table 1
Site-Specific Excision of Plasmid DNA

Recombinase	Constructs transfected		cfu with excision/cfu[a] analyzed[b]	Estimated completion rate[c] (%)	GFP expression[d]
Cre	pPBCre	pNMTCre	29/30	100	+
CinH	pPBCinH	pNMTCinH	29/30	91.8	−
ParA	pPBParA	pNMTParA	29/30	93.3	−
Tn1721	pPBTn1721	pNMTTn1721	29/30	54.8	−
Tn5053	pPBTn5053	pNMTTn5053	27/30	72.6	−
ϕC31	pPBC31	pNMTC31	29/30	87.2	−
TP901-1	pPBTP901	pNMTTP901	29/30	89.4	+
Bxb1	pPBBxb1	pNMTBxb1	29/30	93.5	−
U153	pPBU153	pNMTU153	28/30	27.6	+

[a]cfu = His^+ Leu^+ colony forming units.
[b]Detection of excision by PCR. Data from 10 colonies analyzed for each of three independent experiments.
[c]Based on PCR blot band intensities quantified ($n = 10$) using Scion Image 1.63 program.
[d]Based on visual observation of GFP expression, where "+" indicates observable expression and "−" indicates no expression observed.

for the inversion assay, ***a*** and ***c***, correspond to within P^{NMT} and in the 5′ end of the *eGFP* ORF as shown in Fig. 2a. After recombinase-mediated inversion, these primers are expected to amplify a ~1.6 kb product derived from the *eGFP* inverted construct pPBi-Xinv (Fig. 2c). Primers ***a*** and ***c*** were successful at amplifying a 1.6 kb band when tested on a control plasmid ($pNMTGFP_{rev}$) with the *eGFP* ORF inverted with respect to P^{NMT}.

For the Cre-*lox* and ϕC31 controls, as well as the systems Bxb1, TP901-1, and U153, 90–100% of the colonies analyzed showed site-specific inversion (Table 2). Of particular interest was the lower PCR amplification of the recombination product in the Cre-*lox* when compared with that in ϕC31 system. We believe this reflects the difference between a reversible recombination versus a nonreversible recombination system. With the Cre-*lox* system, the inversion event readily reverts back to the noninverted configuration with the expectation that only half of the molecules would be in the inverted state. In contrast, the ϕC31 system may approach a state of completion where all of the molecules have undergone inversion. By assigning a 50% and 100% estimated completion value to the Cre-*lox* and ϕC31 systems, we estimated the relative completion rates of the other recombination systems and the U153 system (25%) again appeared less efficient than the others (Table 2).

The small serine resolvases had generally been thought not to catalyze inversion. However, the small serine resolvase family appears to be subdivided between the Tn3 and Tn21 subfamilies, with CinH, Tn1721, and Tn5053 in the Tn21 subfamily and ParA in the Tn3 subfamily (Kholodii, 2001). Within the Tn21 subfamily, Tn5053 (Kholodii, 1995) and Tn1721 (Altenbuchner and Schmitt, 1983) have shown low inversion activity in prokaryotic systems at approximately 10% the rate of excision (Altenbuchner and Schmitt, 1983). Our data supports these findings as Tn1721 and Tn5053 mediated inversion was detected in about half of the Leu^+His^+ colonies, and the estimated completion rate in these colonies was ~10% (Table 2). CinH, however, did not show a detectable level of inversion. ParA also failed to show inversion, which appears

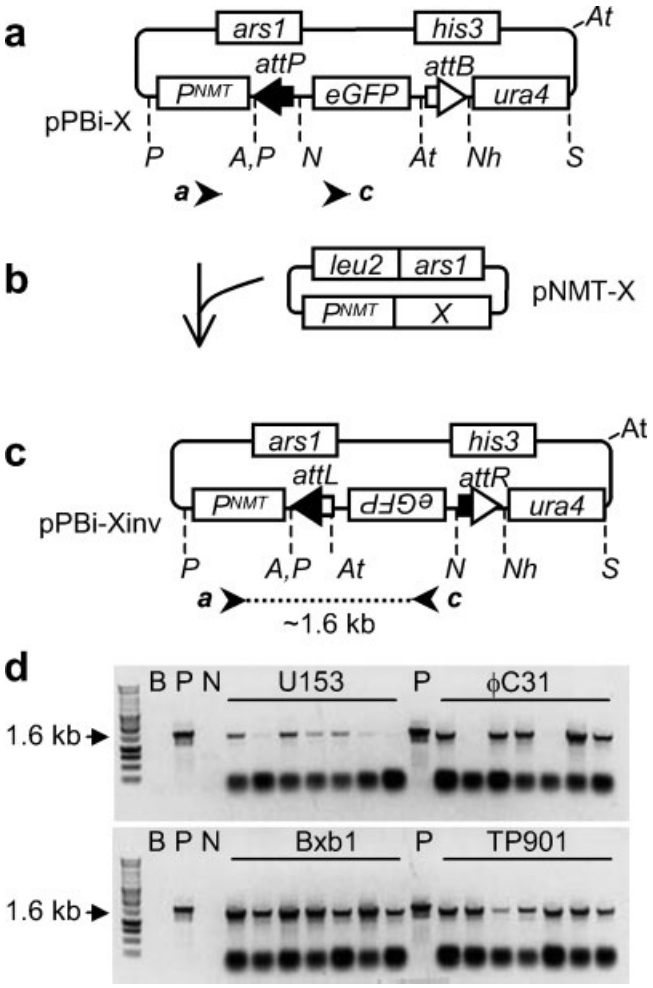

FIG. 2. Site-specific inversion of plasmid DNA. (**a**) pPBi-X contains *eGFP* flanked by sites of recombination system "X" positioned for inversion of *eGFP*. (**b**) Recombinase provided by co-introduced pNMT-X. (**c**) Expected inversion product pPBi-Xinv. PCR primers ***a*** and ***c*** expected to amplify a PCR product of ~1.6 kb. (**d**). PCR reactions on representative Leu^+His^+ colonies from U153, ϕC31, Bxb1, and TP901-1 mediated inversion. Lanes: B, blank; P, positive control (from $pNMTGFP_{rev}$); N, negative control pPBi-X without transformation of pNMT-X. Endonuclease sites as in Fig. 1.

Table 2
Site-Specific Inversion of Plasmid DNA

Recombinase	Constructs transfected		cfu with inversion/ cfu[a] analyzed[b]	Estimated completion rate[c] (%)
Cre	pPBiCre	pNMTCre	24/24	50
CinH	pPBiCinH	pNMTCinH	0/24	0
ParA	pPBiParA	pNMTParA	0/24	0
Tn1721	pPBiTn1721	pNMTTn1721	12/24	10
Tn5053	pPBiTn5053	pNMTTn5053	15/24	10
ϕC31	pPBiC31	pNMTC31	22/24	100
TP901-1	pPBiTP901	pNMTTP901	24/24	100
Bxb1	pPBiBxb1	pNMTBxb1	24/24	100
U153	pPBiU153	pNMTU153	22/24	25

[a]cfu: His^+ Leu^+ colony forming units.
[b]Detection of inversion by PCR. Data from eight colonies analyzed for each of three independent experiments.
[c]Based on PCR blot band intensities quantified ($n = 8$) using Scion Image 1.63 program.

consistent with it being assigned to the Tn3 subfamily that was previously shown incapable of DNA inversion (Grindley *et al*., 1982).

Site-Specific Co-Integration of Plasmid DNA in *S. pombe*

The data above show the ability of these recombination systems to perform intramolecular recombination. To test for intermolecular recombination, two plasmids, pHisB-X and pLeuP-X, where "X" denotes the particular recombination system (Fig. 3a), were tested for co-integrate formation (Fig. 3c). The acceptor construct pHisB-X contains a recombination site (e.g. *attB*) upstream of the *ura4* ORF but lacks the P^{NMT} promoter. It also contains *his3* for selection, and *ars1* for autonomous replication in *S. pombe*. The donor construct pLeuP-X contains the complementary recombination site (*e.g. attP*) inserted into a modified pNMT-TOPO vector that has *leu2* for selection, but lacks *ars1* for replication. For *S. pombe* to become leucine prototrophic, pLeuP-X may be maintained in the cell by incorporating into a host chromosome, or by integrating into the replication proficient plasmid pHisB-X. To provide the recombinase for a possible co-integration event, a third construct was co-introduced. This construct, derived from pNMT-X, was modified to remove both the *leu2* selectable marker, and the *ars1* replication region (Fig. 3b). Hence, the modified construct, pNMTAS-X, was intended to provide transient expression of a recombinase gene.

The PCR assay was identical to that used for the excision test described in Fig. 1. The control systems, Cre-*lox* and ϕC31, performed as expected in this assay, with nearly every His^+Leu^+ transformant yielding a PCR product indicative of co-integrate formation, although this analysis does not reveal what percentage of the molecules recombined (Table 3). With the Cre-*lox* system, both wild type *lox* sites (*loxP*) and mutant sites with a more unidirectional recombination reaction were used (Albert *et al*., 1995; Thomson *et al*., 2003), but a noticeable difference was not found.

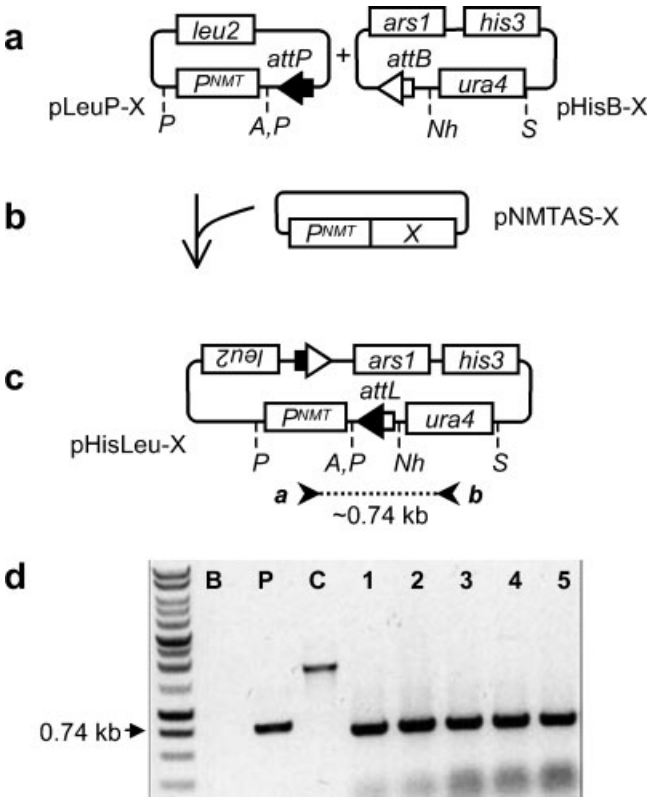

FIG. 3. Site-specific co-integration of plasmid DNA. (**a**) pHisB-X with recombination site (e.g. *attB*) upstream of *ura4* ORF, *his3* for selection, and *ars1* for autonomous replication. pLeuP-X contains P^{NMT}, a downstream recombination site (e.g. *attP*), *leu2* for selection, but devoid of *ars1*. (**b**) Recombinase provided by pNMTAS-X that lacks *leu2* and *ars1*. (**c**) Expected co-integration product pHisLeu-X. PCR primers ***a*** and ***b*** amplify a ~0.74 kb band upon site-specific recombination that fuses P^{NMT} to *ura4*. (**d**) PCR analysis from Bxb1 recombination system. Lanes: B, blank; P, plasmid positive control for integration; C, control transformation of pPB-Bxb1; lanes 1–5, representative Leu^+His^+ colonies. Endonuclease sites as in Fig. 1.

In this co-integration assay, constructs of the Bxb1, TP901-1, and U153 systems produced His^+Leu^+ colonies that also yielded a ~0.74 kb PCR product corresponding

Table 3
Site-Specific Co-Integration of Plasmid DNA

Recombinase	Constructs transfected			$His^+ Leu^+$ cfu[a]	cfu with cointegration junction/cfu analyzed[b]
Cre	pHisBCre	pLeuPCre	pNMTASCre	+	19/19
CinH	pHisBCinH	pLeuPCinH	pNMTASCinH	−	0/19
ParA	pHisBParA	pLeuPParA	pNMTASParA	−	0/19
Tn1721	pHisBTn1721	pLeuPTn1721	pNMTASTn1721	−	0/19
Tn5053	pHisBTn5053	pLeuPTn5053	pNMTASTn5053	−	0/19
ϕC31	pHisBC31	pLeuPC31	pNMTASC31	+	19/19
TP901-1	pHisBTP901	pLeuPTP901	pNMTASTP901	+	19/19
Bxb1	pHisBBxb1	pLeuPBxb1	pNMTASBxb1	+	19/19
U153	pHisBU153	pLeuPU153	pNMTASU153	+	6/19

[a]cfu: His^+ Leu^+ colony forming units; "−" indicates ≤ background rate (without recombinase plasmid control); "+" indicates > background rate.
[b]PCR detection of co-integrate junction. Data from 3 independent experiments, with 6–7 colonies analyzed per experiment.

to the recombination junction. In the case of U153, however, only a third of the colonies were positive for co-integration. For the small serine resolvase systems CinH, ParA, Tn1721, and Tn5053, the inclusion of pNMTAS-X failed to yield His^+Leu^+ transformant over the background rate of His^+Leu^+ colonies without pNMTAS-X (Table 3). Nor did PCR reveal a recombination junction. This indicates a lack of co-integrate plasmid formation, or a highly unstable reaction that leads back to excision, followed by the loss of the *leu2* donor plasmid. The latter interpretation is not likely given that use of reversible wild type *lox* sites in the control Cre-*lox* experiments showed detectable co-integration formation.

Site-Specific Excision of Nuclear DNA from *S. pombe* Genome

The tests described earlier detected site-specific recombination in *S. pombe*, but the events may or may not have occurred in the nucleus. We therefore designed experiments to test for site-specific recombination in the *S. pombe* genome. The pRLPB-X vectors (Fig. 4a), where "X" denotes the particular recombination system, were constructed with the same P^{NMT}*-attP-eGFP-attB* fragments as described in the pPB-X series (Fig. 1a) but with the downstream ORF from *bsd* (blasticidin resistance gene) instead of *ura4* (Fig. 4a). The constructs lack *ars1* for autonomous replication but contain *ura4* gene for homologous insertion into the *ura-294*$^-$ mutant locus of *S. pombe* Sp223 (Fig. 4b). $Ura4^+$ colonies were examined by Southern blotting for single-copy insertion of pRLPB-X. A *ura4* ORF probe detects a 6.8 kb fragment in the wild type genome, but 3.4 and 10.2 kb fragments upon the homologous insertion of pRLPB-X (Fig. 4b, c, f). Representative single copy insertions of pRLPB-X were then tested for recombinase-mediated excision of the *eGFP* marker. The recombinase was provided by a second round of transformation with pNMT-X (Fig. 4d) and individual Leu^+Ura^+ colonies were assayed by PCR for conversion of a ~1.6 kb band before recombination to a ~0.80 kb band after recombination (Fig. 4c,g). Table 4 summarizes the data from three independent experiments. With each of these systems, 77–100% of colonies examined scored positive for excision with the PCR assay. This indicates that each of the recombinases can enter the nucleus and perform site-specific excision on chromosomal DNA.

Genomic DNA from 10 representative clones that scored positive by the PCR assay was examined by Southern blotting. DNA was cleaved with *Pst*I and probed with *bsd* DNA. A 1.5 kb band or a 0.7 kb band was found before or after recombination, respectively. This size change is consistent with the loss of *eGFP* and one recombination site (Fig. 4c,e,h). On the basis of the relative intensities of the 1.5 kb parental and the 0.7 kb recombination junction bands, the efficiency of recombination ranged from 66 to 100% for recombinase CinH, ParA, Bxb1, and TP901-1 (Table 4). The systems Tn1721 and Tn5053 were much lower at 22% and 28%, respectively. With the U153 system, the Southern blots of Leu^+Ura^+ colonies could not detect the presence of the recombination junction, even though the junction was found by PCR. This suggested that only a subset of the cells within the colonies had undergone site-specific recombination. Since the previous data show that U153 recombination site does not interfere with expression of the downstream ORF, representative U153 Leu^+Ura^+ colonies were grown in the presence of blasticidin, and 40/40 colonies survived selection. The Southern blots of all 10 representative blasticidin resistant clones revealed the expected hybridization pattern for site-specific excision. This demonstrates that U153 is also capable of site-specific excision of nuclear DNA (Fig. 4h).

Site-Specific Integration of DNA into the *S. pombe* Genome

As shown earlier, Bxb1, TP901-1, and U153 catalyzed plasmid–plasmid co-integration. To test whether these systems would integrate introduced DNA into the host chromosome, target sites were engineered into the *ura4-294* locus. Each of these pRLBZ-X constructs, where "X" denotes the particular recombination system (Fig. 5a), comprises an attachment site (*attB*) upstream of a *bsd* coding region devoid of its promoter, followed downstream by the 1.8 kb $ura4^+$ gene. Ura^+ colonies

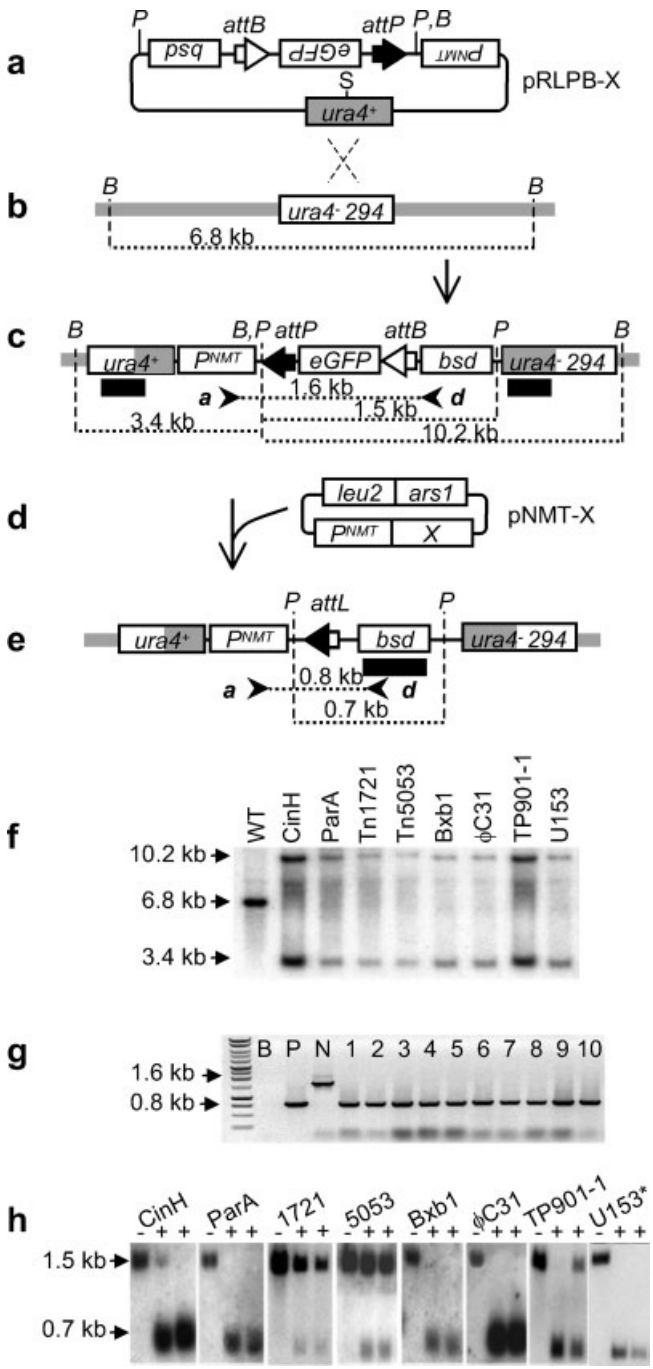

FIG. 4. Site-specific excision of nuclear DNA. (**a**) pRLPB-X with *ura4*$^+$ for homologous integration and selection. (**b**) Structure of *ura4-294* locus. (**c**) Structure after pRLPB-X insertion into *ura4-294*. (**d**) pRLPB-X target lines transformed with pNMT-X. (**e**) Structure from pNMT-X promoted excision of *eGFP*. (**f**) Southern blot of *Bam*HI (*B*) cleaved pRLPB-X target line DNA probed with ^{32}P *ura4*. (**g**) PCR analysis with primers ***a*** and ***d***. Lanes: B, blank; P, positive control; N, negative control without pNMT-X; Lanes 1–10, representative Leu$^+$Ura$^+$ colonies from Bxb1 system. (**h**) Southern blot of genomic DNA cleaved with *Pst*I (*P*) and probed with ^{32}P *bsd*. *DNA from U153-derived colonies after additional blasticidin selection.

that integrated a single copy of pRLBZ-X by homologous insertion into the *ura4-294* locus were identified by DNA hybridization where a *ura4* ORF-hybridizing *Bam*HI fragment shifts from 6.8 to 11.2 kb (Fig. 5b,c,f). These "target" lines were then transformed with pLeuP-X and pNMTAS-X (Fig. 5d). Recombinase produced by pNMTAS-X would promote site-specific integration of pLeuP-X into the genomic *attB*, leading to the genomic structure depicted in Figure 4e. Individual Leu$^+$ Ura$^+$ colonies were analyzed by PCR for the presence of a 0.8 kb band indicative of the joining between the P^{NMT} and *bsd* DNA (Fig. 4e,g).

PCR results indicate that Bxb1, TP901-1, U153 and the control ϕC31 systems were capable of facilitating site-specific integration into the genomic *attB*, leading to the genomic structure depicted in Fig. 5e. Table 5 summarizes the data from 4 independent experiments. On the basis of the PCR assay that detects the ~0.8 kb recombination junction, 55–85% of the colonies scored positive for site-specific integration. Those scored negative were presumed to have pLeuP-X integrated elsewhere in the genome. Consistent with this interpretation was that the recovery of Leu$^+$Ura$^+$ colonies was not significantly higher that the control experiments without pNMTAS-X. This suggests that site-specific integration was occurring at about the same rate as nonsite-specific integration of pLeuP-X into the *S. pombe* genome. Since pLeuP-X contains a *leu2* gene, it is possible that some percentage of these colonies might have harbored a copy of pLeuP-X at the *leu* locus. However, we have not investigated this possibility.

Genomic DNA from 10 Leu$^+$ Ura$^+$ colonies for each of the recombination systems was cleaved by *Eco*RI and hybridized to labeled *bsd* DNA. In wild type Sp223, *bsd* hybridization was not detected. In the target lines, the probe detected a 6.5 kb band. After site-specific integration, a 0.7 kb band was observed (Fig. 5c,e,h). However, in most instances the parental 6.5 kb band was still present, suggesting that the DNA was derived from a heterogenous population of cells, consisting of both parental and site-specific integrant types. In the case of Bxb1, for example, 60% of the colonies examined still showed the parental 6.5 kb band. Once again, with the U153 system, the 0.7 kb hybridizing band was not detected on Leu$^+$Ura$^+$ colonies until these colonies were purified through blasticidin selection, in which 10/10 clones showed targeted insertion in Southern blots. Despite the blasticidin selection, however, the parental 6.5 kb band is seen, indicating that these colonies are not homogenous for the site-specific integration event.

The pLeuP-X constructs contain a 188 bp fragment of the SV40 promoter upstream of *leu2* that can serve as a hybridization tag to investigate the possible co-integration of extra copies of pLeuP-X at non-specific locations. Southern blot analysis using this SV40 promoter fragment (not shown) revealed that 1 of 10 Bxb1 integrants, 1 of 9 TP901-1 integrants, and 2 of 10 U153 or ϕC31 integrants showed an extra random insertion of pLeuP-X DNA.

Table 4
Site-Specific Excision of Nuclear DNA

Recombinase	Constructs transfected		$Ura^+ Leu^+$ cfu[a]	Ura^+Leu^+ cfu/total cfu (10^{-4})[a]	cfu with excision/cfu analyzed[b]	Estimated completion rate[c] (%)
CinH	pRLPBBCinH	pNMTCinH	533 ± 214	1.5 ± 0.94	23/30	95.0
ParA	pRLPBBParA	pNMTParA	359 ± 302	0.81 ± 0.49	29/30	96.7
Tn1721	pRLPBB1721	pNMTTn1721	301 ± 259	2.2 ± 1.9	25/27	21.5
Tn5053	pRLPBB5053	pNMTTn5053	792 ± 769	2.3 ± 2.6	25/28	27.7
ϕC31	pRLPBBC31	pNMTC31	735 ± 383	1.4 ± 1.1	23/29	91.4
TP901-1	pRLPBBTP901	pNMTTP901	2240 ± 360	3.7 ± 3.2	30/30	66.0
Bxb1	pRLPBBBxb1	pNMTBxb1	89.6 ± 60.3	0.89 ± 0.81	30/30	100
U153	pRLPBBU153	pNMTU153	1320 ± 332	20 ± 10	15/17	(100[d])

[a]cfu: Ura^+Leu^+ colony forming units; mean ± SD from three independent experiments.
[b]Detection of excision by PCR. Data from 10 colonies analyzed for each of three independent experiments after $Ura^+ Leu^+$ selection.
[c]Based on Southern blot band intensities quantified ($n = 10$) using Scion Image 1.63 program after $Ura^+ Leu^+$ selection.
[d]Based on Southern blot band intensities quantified ($n = 10$) using Scion Image 1.63 program after $Ura^+ Leu^+$ and blasticidin selection.

Specificity of Recombination in *S. pombe*

Future use of site-specific recombination systems for genome engineering may require the presence of several different recombination systems within a transgenic locus. To confirm that the different recombinases would not cross react with heterologous recombination sites, the plasmid-based deletion assay described earlier was tested with heterologous recombinases. Within the small serine resolvase subfamily, when a pPB-X construct was tested with a heterologous pNMT-X construct, site-specific deletion was not observed (data not shown). The same held true for the recombinases from the large serine resolvase subfamily (data not shown), suggesting that the recombinase/recombination site specificity seen within the prokaryotic environment is maintained in a eukaryotic cell.

Effect of Recombinase Overexpression on Growth in *S. pombe*

Recombinases are DNA binding proteins and in theory, the possibility exists that hyper-production of such proteins may result in excessive DNA binding or DNA scanning. Additionally, should cryptic recombination sites be present in the host genome, there may be unintended recombination of host DNA. The possibility that such events have a detrimental effect on the cell was examined. Cultures of *S. pombe* harboring each of the pNMT-X constructs of CinH, ParA, Tn1721, Tn5053, Bxb1, ϕC31, TP901-1, and U153 were compared with those with an empty vector control (pNMT-EV, the same vector backbone without a recombinase gene) and to a construct expressing *eGFP* (pNMT-*eGFP*). Compared with the empty vector control, or to the *eGFP* construct, the cultures producing these recombinases grew at comparable rates (data not shown). The conclusion can be made that these recombinases do not adversely affect cell growth, at least with *S. pombe*, and by inference, are not likely to cause deleterious effects on the host genome.

Future Prospects

Like the Cre-*lox*, FLP-*FRT*, ϕC31-*att* and other systems, the seven bacterial recombination systems described in this study should be suitable for the precise genetic modification of eukaryotic genomes. In the cases of TP901-1, Bxb1, and U153 site-specific co-integration in mammalian cells has recently been shown (Hollis et al., 2003; Keravala et al., 2006; Russell et al., 2006; Stoll et al., 2002), although with the report of Bxb1 and U153, the recombination was demonstrated with extrachromosomal DNA, which might not necessarily reflect its activity in the chromosome. Nevertheless, this is not to say that extrachromosomal events are without merit. Aside from providing a first indication of function, there are certain applications that use extrachromosomal DNA as substrate. For instance, a method for plant-viral based production of pharmaceutical proteins uses site-specific recombination of extrachromosomal DNA to assemble the full-length virus in planta. This permits a rapid assortment of different viral promoters with different genes to be expressed (Marillonnet *et al*., 2004).

In this work, we also show that the described recombination systems can perform site-specific recombination in eukaryotic chromosomes. The CinH, ParA, Tn1721, and Tn5053 systems are dedicated deletion systems, and are ideally suited for removing selectable marker genes or other unneeded DNA from eukaryotic cells, including the removal of nearly all exogenously introduced DNA (save a recombination site) from a transgene locus. The availability of additional deletion systems could greatly aid genetic analysis. For example, knock-out mice could be configured to inactivate multiple genes, each controlled by a unique recombination system. Tn1721 and Tn5053 also have low inversion activity and could be used, for instance, to invert DNA to control gene expression. Of particular significance is that these 4 recombination systems require recombination targets much larger than that of the Cre-*lox* system. Unlike the relatively small 34 bp *lox* or *FRT* sites, the recombination sites of these four systems range from 113 to 176 bp. The larger-size requirement for target specificity may lessen the probability of unintended recombination with native host sequences that resemble the intended target. Furthermore, the small serine resolvases are incapable of mediating integration and would therefore be unable to re-integrate DNA into the host genome after excision. Although this would seem unlikely,

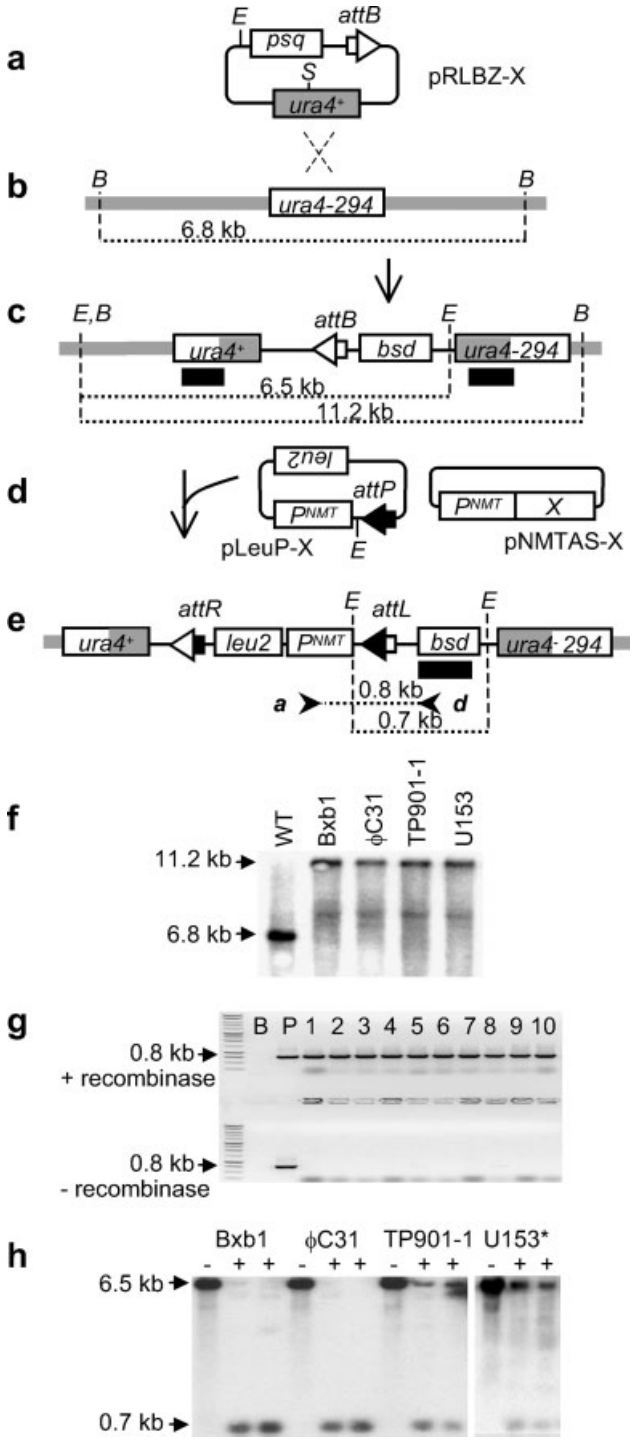

FIG. 5. Site-specific integration into nuclear DNA. (**a**) pRLBZ-X with *attB* upstream of promoterless *bsd*. (**b**) Structure of *ura4-294* locus. (**c**) Expected structure after pRLBZ-X homologous integration into *ura4-294*. (**d**) pRLBZ-X target lines transformed with pLeuP-X and pNMTAS-X. (**e**) Structure from pNMTAS-X promoted integration of pLeuP-X. (**f**) Southern blot of pRLBZ-X target line DNA cleaved with *Bam*HI (*B*) and probed with ^{32}P *ura4* DNA. (**g**) Representative PCR analysis from Bxb1 system from Primers ***a*** and ***d***. (**h**) Southern blot of recombinase-mediated integration. ^{32}P *bsd* probe detected a shift in *Eco*RI (*E*) cleaved genomic DNA after site-specific insertion of pLeuP-X. *DNA from U153-derived colonies after additional blasticidin selection.

the presence of excised DNA in the wheat genome has been observed with use of the Cre-*lox* system, leading to suggestions that on rare occasions, the excised molecule can be propagated through cycles of reinsertion into its original genomic location (Srivastava and Ow, 2003).

The Bxb1, TP901-1, and U153 systems are capable of excision, inversion, and integration reactions. Since the reactions are unidirectional, these systems could be used, for instance, to invert DNA to control gene expression. Also, like the ϕC31 system, Bxb1, TP901-1 and U153 are ideally suited for integrating DNA into the host genome by targeting a transgenic recombination site, or a native host sequence that functionally resembles the complementary recombination site (Hollis *et al*., 2003). The reaction should be stable since the integrated molecule will not be re-excised by the recombinase protein without an additional excisionase cofactor.

In several reports on the use of ϕC31 or similar systems such as TP901-1 and R4, studies were conducted using an *attP* as a genomic target (Belteki et al., 2003; Olivares et al., 2001; Stoll et al., 2002; Thomason et al., 2001; Thyagarajan et al., 2001). Host genome encoded "pseudo" *attP* sites (partial identity to *attP*) were discovered that facilitated integration into the mouse and human genomes. In the current study, we integrated molecules containing an *attP* site into the host genome that contains an *attB* site, analogous to the report in which the ϕC31 *attB* site introduced into the tobacco chloroplast genome served as a target for the insertion of *attP*-containing plasmids (Lutz *et al*., 2004), or a more recent report in which the A118 recombination system directed the integration of DNA into pseudo *attB* sites in the human genome (Keravala *et al*., 2006). Although it remains to be tested with each recombination system, it seems most probable that the large resolvase family of recombination systems can target either *attP* or *attB* sites for the insertion of new DNA.

To conclude, we view this work as a guide for others to further optimize these recombination systems for their particular needs. Recombination efficiency depends on a combination of factors, ranging from differences in cell (species) type, target site position effects, recombinase protein activity, and recombinase gene expression. Among these factors, the latter is most readily amenable to improvement by the investigator through changes of transcriptional and post-transcriptional signals, including the alteration of codons for species-optimized expression. So far, derivatives of the *S. pombe* test constructs (Fig. 1a) in *Arabidopsis* indicate that these recombination systems are functional in *planta*, and they also follow the same trend in relative recombination efficiency (ParA, CinH, Bxb1 ≫ Tn1721, Tn5053, U153). Additional tests are currently underway with promoter and codon-optimized recombinase genes that may improve further the utility of these systems.

MATERIALS AND METHODS

Biological Materials

S. pombe strains: FY527 (*h*$^-$ *ada6-M216 his3-D1 leu-32 ura-D18*) was obtained from S. Forsburg. Sp223 (*h*$^-$

Table 5
Site-Specific Integration into Nuclear DNA

Recombinase	Constructs transfected			$Ura^+ Leu^+$ (10^2)[a]	Ura^+Leu^+ cfu/total cfu (10^{-4})[a]	cfu with integration/cfu analyzed[b]	Site-specific targeting increase over background[c] (%)
φC31	pPLBZC31	pLeuPC31	pNMTASC31	1.6 ± 1.9	0.34 ± 0.2	38/40	1.9
TP901-1	pRLBZTP901	pLeuPTP901	pNMTASTP901	4.0 ± 2.7	4.2 ± 10	31/40	1.8
Bxb1	pRLBZBxb1	pLeuPBxb1	pNMTASBxb1	13 ± 19	3.5 ± 10	34/40	2.3
U153	pRLBZU153	pLeuPU153	pNMTASU153	5.9 ± .94	1.8 ± 1.8	22/40	1.4

[a]cfu: Ura^+Leu^+ colony forming units.
[b]PCR detection of co-integration junction. Data from four independent experiments after Ura^+ Leu^+ selection.
[c]Increase in site-specific integration over background determined by dividing the average count of total Ura^+ Leu^+ colonies with recombinase present by the average count of Ura^+ Leu^+ colonies without recombinase present.

leu1.32 ura4.294 ade.216) has been described previously (Ortiz *et al.*, 1992). Recombinase genes were obtained from Gennady Kholodii (CinH, pKLH205.63; ParA, pWIS17; Tn1721, pRU576.8; and Tn5053, pKLH53.6), Richard Calendar (U153, genomic DNA of *Listeria monocytogenes* phage phiCU-SI153/95), Graham Hatfull (Bxb1, pMA1), and Karen Hammer (TP901-1, pBC170).

DNA Constructs

See supplemental materials for plasmid maps and oligonucleotide sequences used for PCR and assembly of recombination sites.

pPB-Cre is derived from pLT43 (Thomason *et al.*, 2001), which contains *his3* and *ars1* for *S. pombe* selection and replication, respectively, and the thiamine repressible promoter P^{NMT} for expression of downstream genes. The *Not*I site of pLT43 was removed by digest, DNA polymerase I, with blunt ends closed by DNA ligase. The *Sac*I flanked φC31 ORF was replaced with a first *lox* site flanked by *Asc*I and *Not*I, leaving the downstream *Sac*I site intact. The construct was then cleaved with *Not*I and *Sac*I for insertion of a second *lox* site that contains flanking *Bcl*I and *Nhe*I sites. An *ura4* fragment included a 5′ *Nhe*I site and a 3′ *Sac*I site 3′ for placement between the *Nhe*I and *Sac*I downstream of the second *lox* site. An *eGFP* fragment included a 5′ *Not*I site and a 3′ *Eag*I site was inserted into the *Not*I site between the first and second *lox* sites to yield pPB-Cre.

The pPB-X constructs, (Fig. 1a, S1), where X represents the recombination system used, were derived from pPB-Cre with system-specific recombination sites instead of the *lox* sites, shown in Fig. 1a, S1. System-specific recombination sites were made with flanking *Asc*I and *Not*I sites for directional replacement of the first (upstream) *lox* site, or with flanking *Bcl*I and *Nhe*I sites for directional replacement of the second (downstream) *lox* site.

The pNMT-X constructs (Fig. 1b, S2) contain the pNMT1-TOPO (Invitrogen) backbone. The system-specific recombinase genes were amplified by Turbo Pfu (Stratagene), treated with Taq polymerase to add overhanging A's to the ORFs, and inserted into pNMT1-TOPO.

The pPBi-X constructs (Fig. 2a, S3) were derived from pPB-X. Recombination site in the reverse orientation relative to the corresponding site between *eGFP* and *ura4* in pPB-X were made with *Bcl*I and *Nhe*I flanking sites for replacement of the corresponding site in pPB-X.

The pHisB-X constructs (Fig. 3a, S4) were derived from the corresponding pPB-X constructs by removal of the P^{NMT}*-attP-eGFP* fragment through treatment with *Pst*I and *Bcl*I, DNA polymerase I, and DNA ligase.

pLeuP-X constructs (Fig. 3a, S5) were generated by first inserting a recombination site into a pNMT-TOPO derivative that had been modified to contain unique *Asc*I and *Not*I sites downstream of P^{NMT}. This was followed by cleavage by *Sal*I and *Afe*I to remove *ars1*, polymerase I treatment, and ligation to form pLeuP-X.

The pNMTAS-X constructs (Fig. 3b, S6) were generated by removal of *leu2* and *ars1* sequences from pNMT-X constructs through cleavage with *Afe*I and *Stu*I, followed by self ligation.

The pRLPB-X constructs (Fig. 4a, S7) were derived from pREP4X (Forsburg, 1993; ATCC 87604) by cleaving with *EcoR*I and replacing *ars1* with a synthetic linker consisting of *Eco*RI-*Asc*I-*Bgl*II-*Nhe*I-*Sac*I-*EcoR*I. A *bsd* ORF was inserted between *Nhe*I and *Sac*I to form pRLB. An *Asc*I-*Pst*I-*attP-eGFP-attB-Nhe*I fragment from the pPB-X was then inserted between the *Asc*I and *Nhe*I sites of pRLB to form pRLPB-X.

The pRLBZ-X constructs (Fig. 5a, S8) were derived from pRLB by inserting a *Bcl*I, *Aat2-attB-Nhe*I fragment between the *Bgl*II and *Nhe*I sites of pRLB; which was further modified by cleavage with *Asc*I and *Afl*II to remove the sequence corresponding to primer ***a*** of P^{NMT}, followed by treatment with DNA polymerase I and ligase.

$pNMTGFP_{rev}$ was generated by PCR synthesis of the *eGFP* ORF followed, insertion into pNMT-TOPO, and screening for the antisense orientation with respect to P^{NMT}.

S. pombe Transformation

Plasmids were introduced by the cold sorbitol electroporation procedure (Forsburg lab; http://www-rcf.usc.edu/~forsburg/tfmn.html). For homologous insertion of pRLPB-X or pRLBZ-X into the Sp223 genome, *Stu*I cleaved linear DNA with $ura4^+$ was introduced using

lithium acetate protocol as described (www.umanitoba.ca/faculties/medicine/biochem/gietz/Trafo.html).

Molecular Analysis

Individual yeast colonies were retrieved from plates and added directly to a tube for PCR amplification. Sequences of primers ***a***, ***b***, ***c***, and ***d*** shown in the Figures are described in supplemental materials. Genomic DNA was extracted using the "Gentra Genomic DNA extraction Kit" following the 10 ml protocol. DNA was purified twice before 10 μg of genomic DNA was cleaved with *Bam*HI, *Eco*RI, or *Pst*I. Southern hybridizations were conducted with ^{32}P-labeled DNA probes of PCR-amplified *ura4* or *bsd* DNA. Digitalized images with a resolution of 200 dpi in black on white background TIF format were analyzed using the density measurement option of the Scion Image 1.63 program at http://www.scioncorp.com/pages/download_now.htm.

ACKNOWLEDGMENTS

We are grateful to R. Calender, S. Forsburg, K. Hammer, G. Hatfull, and G. Kholodii for providing biological materials, and S. Wei for technical assistance.

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